Effects of Endocrine-disrupting Contaminants on Amphibian Oogenesis: Methoxychlor Inhibits Progesterone-induced Maturation of *Xenopus laevis* Oocytes *in Vitro*

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There is currently little evidence of pollution-induced endocrine dysfunction in amphibia, in spite of widespread concern over global declines in this ecologically diverse group. Data regarding the potential effects of endocrine-disrupting contaminants (EDCs) on reproductive function in amphibia are particularly lacking. We hypothesized that estrogenic EDCs may disrupt progesterone-induced oocyte maturation in the adult amphibian ovary, and tested this with an *in vitro* germinal vesicle breakdown assay using defolliculated oocytes from the African clawed frog, *Xenopus laevis*. While a variety of natural and synthetic estrogens and xenoestrogens were inactive in this system, the proestrogenic pesticide methoxychlor was a surprisingly potent inhibitor of progesterone-induced oocyte maturation (median inhibitive concentration, 72 nM). This inhibitory activity was specific to methoxychlor, rather than to its estrogenic contaminants or metabolites, and was not antagonized by the estrogen receptor antagonist ICI 182,780, suggesting that this activity is not estrogenic per se. The inhibitory activity of methoxychlor was dose dependent, reversible, and early acting. However, washout was unable to reverse the effect of short methoxychlor exposure, and methoxychlor did not competitively displace [^{3}H]progesterone from a specific binding site in the oocyte plasma membrane. Therefore, methoxychlor may exert its action not directly at the site of progesterone action, but downstream on early events in maturational signaling, although the precise mechanism of action is unclear. The activity of methoxychlor in this system indicates that xenobiotics may exert endocrine-disrupting effects through interference with progestin-regulated processes and through mechanisms other than receptor antagonism. *Key words*: amphibia, antiprogestin, endocrine disruptors, estrogen, GVBD, methoxychlor, oocyte maturation, progesterone, xenobiotics, *Xenopus*. *Environ Health Perspect* 107:285–292 (1999). [Online 10 March 1999]
http://ehpnet1.niehs.nih.gov/docs/1999/107p285-292pickford/abstract.html

In spite of increasing alarm over global population declines in amphibian species over the last 20 years, little evidence of reproductive toxicity of xenobiotics in amphibians is available. Population declines in a variety of amphibian groups have been documented worldwide, although the etiology of these declines remains unclear. Habitat destruction, fragmentation, or disturbance may be sufficient to explain some declines, but not all. Other potential causes include habitat acidification, predation/competition by introduced species (*1*), increased ultraviolet (UV) radiation resulting from atmospheric ozone depletion (*2*), and exposure to toxic environmental contaminants (*3,4*). Amphibians may be particularly vulnerable to waterborne environmental contaminants due to their largely aquatic life histories and their highly permeable skin (*5*).

A number of man-made environmental pollutants have the potential to interfere with endocrine function (*6–11*), and there is evidence of reproductive or endocrine dysfunction in wildlife species that have been exposed environmentally to such endocrine-disrupting contaminants (EDCs) (*12–18*). While there have been recent reports of alterations of the stress hormone axis in amphibia (*19,20*), there is little or no published evidence of reproductive dysfunction in this group as a result of exposure to EDCs. A recent special report on environmental endocrine disruption by the U.S. EPA cited no reports of such effects in amphibians, although it concluded that "...this class of vertebrate might represent a unique sentinel animal model for laboratory and field exposure studies" (*21*).

Carey and Bryant (*4*) noted that environmental toxicants may affect amphibian populations in a number of ways. Contaminants may kill individuals, either directly (e.g., mortality of western spotted frogs after DDT spraying) (*22*) or indirectly (e.g., through alterations in immune or neurological function) (*23*). Contaminants may also affect recruitment in amphibian populations by disrupting normal growth and development of the young or by impairing adult reproduction (*4*).

Female reproductive function could be affected by EDCs at a number of target sites including the brain, pituitary, gonad, liver, and oviduct. Gonadal effects of EDCs have considerable potential to impair the reproduction of female amphibia and have been reported in other lower vertebrate wildlife groups. Female juvenile alligators from the pesticide-contaminated Lake Apopka, Florida, exhibit a number of ovarian abnormalities including high frequencies of polynuclear oocytes and polyovular follicles (*12*), suppressed synthesis of 17β-estradiol (E_2), and reduced aromatase activity *in vitro* (*14,24*). These abnormalities presumably represent organizational effects of EDCs on the developing gonad, resulting from embryonic or neonatal exposure. Whereas these organizational alterations in the structure and/or function of reproductive tissues may have the greatest potential impact on the reproductive fitness of a population (*25*), activational effects of EDCs such as the modulation of endocrine signaling in the adult gonad may also significantly impair reproduction. Polyaromatic hydrocarbons (PAHs) have been shown to impair various aspects of ovarian function in adult fish. PAHs inhibited oocyte growth, caused increased follicular atresia, and prevented final oocyte maturation in the Atlantic croaker (*26*). Furthermore, the organochlorine compounds kepone and *o,p'*-DDD inhibited *in vitro* final maturation of Atlantic croaker oocytes, which is induced by the steroid 17α,20β,21-trihydroxy-4-pregnen-3-one (20β-S) (*27*).

Oocyte maturation is the final phase of oogenesis, which involves the release of meiotic prophase I arrest, allowing the oocyte to advance to metaphase II. Maturation in amphibia, which results in germinal vesicle breakdown (GVBD), spindle formation, and extrusion of the first polar body (*28*), is stimulated by progesterone (*29*). Amphibian oocyte maturation may therefore be sensitive to the effects of xenobiotics that have the ability to disrupt sex steroid signaling. Moreover, it has been reported that *in vitro* progesterone-induced GVBD can be inhibited in *Rana pipiens* oocytes by E_2 (*30*) and in *Xenopus* oocytes by 17α-ethinyl estradiol (*28*), suggesting that amphibian oocyte maturation may be sensitive to xenobiotics with estrogenic activity. Oocyte maturation is a prerequisite for subsequent fertilization of the released ova; thus, disruption

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This work was funded by an Award in Ecotoxicology (Prize Studentship) to D.P. from The Wellcome Trust.
Received 20 October 1998; accepted 15 December 1998.

of this process has considerable potential to impair female amphibian reproduction.

We hypothesized that progesterone-induced maturation of amphibian oocytes could be disrupted by environmental pollutants with estrogenic activity. We have tested this hypothesis in an *in vitro* maturation, or GVBD, assay using oocytes from the African clawed frog, *Xenopus laevis*. *Xenopus* is a well-established amphibian model; these animals are easily maintained in captivity, where follicular development in the ovaries of adult females is asynchronous (*31*). Consequently, at any time, the adult *Xenopus* ovary contains follicles at all stages of development, and large preovulatory (Stage VI) oocytes can be obtained throughout the year. Follicle cell-free (denuded, or "naked") oocytes were used in this GVBD assay to assess the potential for direct effects of xenoestrogens on progesterone-induced maturation. This approach excludes the potential for indirect maturational effects of EDCs, for example, through alterations in steroidogenesis, which has been reported for a number of EDCs (*14,18,32*). We have optimized short-term culture conditions for *in vitro* progesterone-induced maturation of Stage VI *Xenopus* oocytes and have tested the ability of natural and synthetic estrogens and a number of xenoestrogens to modulate this process.

Materials and Methods

Experimental animals. Adult female *Xenopus laevis* were purchased from Blades Biological Supplies (Portsmouth, UK) and maintained in opaque white plastic tanks at 20–23°C in dechlorinated water, which was changed biweekly after feeding animals Tetra Reptomin (TetraWerk, Melle, Germany). Frogs were terminally anaesthetized by immersion in 0.2% solution of MS 222 (3-aminobenzoic acid ethylester, methanesulfonate salt; Sigma, Poole, UK) buffered to pH 7 with 0.5 M sodium bicarbonate, followed by destruction of the brain. All animal procedures were in compliance with the Animals (Scientific Procedures) Act 1986.

Hormones and xenobiotics. Progesterone, E_2, 17α-ethinyl estradiol, *o,p′*-DDT, octylphenol, di-*n*-butyl phthalate, bisphenol A (Sigma-Aldrich, Poole, UK); ICI 182,780 {7α-[9-(4,4,5,5,5-pentafluoropentylsulfinyl) nonyl]estra-1,3,5(10)-triene-3,17-β-diol; a gift from Alan Wakeling; Zeneca Pharmaceuticals, Alderly Edge, UK}; RU 486 [mifepristone, 17β-hydroxy-11β-(4-dimethyl-aminophenyl)-17α-(1-propynyl)-estra-4,9-dien-3-one; Roussel-UCLAF, Romansville, France]; and ZK 98.299 [onapristone, 11β-(4-dimethyl-aminopropyl)-17α-hydroxy-17β-(3-hydroxypropyl)-13-methyl-4,9-gonadien-3-one; Schering AG, Berlin, Germany] were dissolved in 100% ethanol. Methoxychlor (95% laboratory grade; Sigma-Aldrich, Poole, UK), purified methoxychlor (99.25%), and HPTE [2,2-bis(*p*-hydroxyphenyl)-1,1-trichloroethane; 99.21%], gifts from William Kelce, U.S. EPA, were dissolved in DMSO. All compounds were dispensed in a constant 5-µl volume (final vehicle concentration 0.25%), except ICI 182,780, which was dispensed in a 2-µl volume (final vehicle concentration 0.1%).

Oocyte preparation and culture. Ovaries were excised and placed in sterile chilled Ca^{2+}/phenol red-free Hanks balanced salts solution (Sigma, Poole, UK) buffered with 10 mM HEPES, pH 7.6, and diluted to 230 milliosmoles (mOsm) with distilled water (Hanks O). Ovarian tissue was then cut into strips, rinsed several times, and incubated overnight at 4°C in Hanks O. Tissue was then digested for 90 min in a 0.2% solution of collagenase D (Boehringer Mannheim GmbH, Mannheim, Germany) in Hanks O until all of the follicle cell layer was removed from the oocytes, as determined by methyl green staining (0.4% methyl green; 6% acetic acid; 2 mM $CaCl_2$) (*33*). After sufficient digestion, the oocytes were rinsed four times in fresh Hanks O and transferred to a disposable 100-mm petri dish (Costar Corporation, Cambridge, MA) containing modified defined nutrient oocyte medium (mDNOM, pH 7.6). This mDNOM was modified from the DNOM developed by Eppig and Dumont (*33*) by replacing gentamycin with penicillin/streptomycin, omitting phenol red due to its weak estrogenic activity (*34*) and adjusting the osmolarity to 230 mOsm with NaCl.

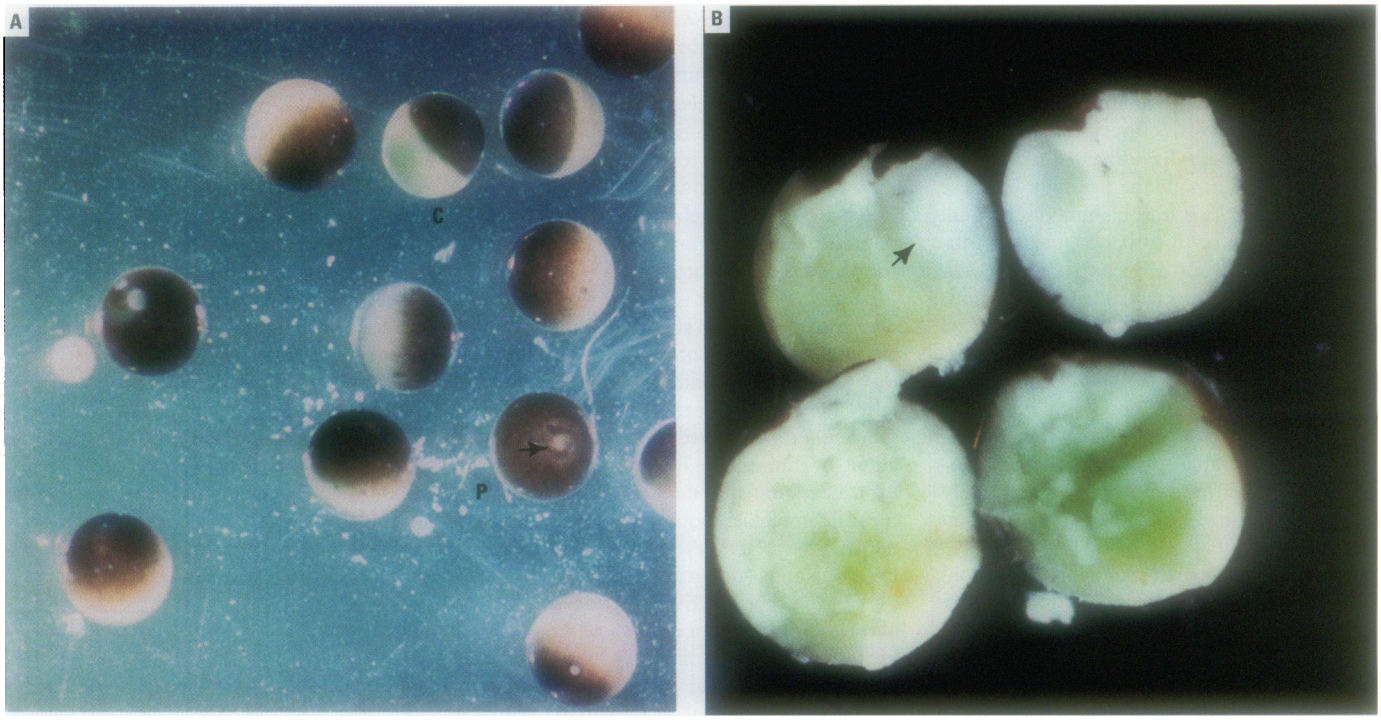

Figure 1. *In vitro* progesterone-induced maturation of *Xenopus laevis* oocytes. (A) Mature (p) and nonmature (c) oocytes 24 hr after exposure to a nonmaximal dose of progesterone. Note the pale "Roux" spot on the animal pole of mature oocytes (arrow). (B) Oocytes fixed with trichloroacetic acid and cracked open. The upper oocyte is nonmature (note germinal vesicle; arrow), whereas the lower oocyte is mature and exhibits germinal vesicle breakdown.

GVBD assay. Large, banded, preovulatory stage VI oocytes were selected by hand using a sterile Pasteur pipette under a dissecting microscope, and plated 20/well in sterile 24-well polystyrene culture plates (Costar Corporation) in 2 ml mDNOM. Progesterone and test compounds at various doses were added at the same time (<10 min apart), except in pretreatment or washout experiments, and mixed by gentle pipetting. Plates were incubated in a shaker at room temperature (20–23°C) for 24 hr; the medium was then aspirated and the oocytes were fixed in 5% (w/v) trichloroacetic acid. Maturation was visible externally as a white "Roux" spot (Fig. 1A) that indicates where the spindle has anchored to the plasma membrane at the animal pole of the oocyte (*35*); GVBD was verified by cracking open the fixed oocyte (Fig. 1B). The maturational response of 20 oocytes in each well was expressed as the percentage exhibiting GVBD, and the mean maturational response for each treatment combination represents a minimum of three replicate wells.

In pretreatment experiments, oocytes were incubated with a test compound before addition of progesterone, without change of culture media. In the washout experiment, oocytes were incubated for 2 hr with methoxychlor and then washed once with fresh media, which was replaced with a further 2 ml of fresh mDNOM before progesterone was added.

Progesterone receptor binding assay. We used a radioreceptor assay to assess the ability of some compounds to interact with the oocyte membrane progesterone receptor (omPR). This assay is based on one described by Liu and Patiño (*36*) in which they characterized and validated high affinity binding of progesterone to a component of *Xenopus* oocyte plasma membrane (OPM). *Xenopus* OPM was prepared as follows. Stage VI oocytes were homogenized in ice-cold oocyte homogenizing buffer (OHB; 83 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.6, 1 mM dithiothreitol, 12 mM monothioglycerol, and 0.5 mM phenylmethylsulfonyl fluoride, sterile filtered) at a ratio of 5 ml OHB/2 g oocytes. This homogenate was centrifuged three times at 1,000*g* for 10 min at 4°C; the yolk/melanosome pellet was discarded each time. The supernatant was then centrifuged three times at 20,000*g* for 30 min at 4°C; each time, the supernatant was discarded and the pellet was resuspended in 10 ml OHB. The final plasma membrane pellet was resuspended in 5 ml OHB/10 ml original homogenate, aliquoted, and flash frozen in liquid nitrogen.

Competitive binding assays to determine the affinity of methoxychlor and HPTE for the omPR were performed with OPM as follows. Competitor compounds (dissolved in DMSO) were dispensed in 3- µl volumes into 12 × 75 mm borosilicate glass tubes and mixed with 150 µl radioactive tracer (120,000 cpm/tube, 1,2,6,7-^{3}H(N)-progesterone; NEN Life Science Products Inc., Hounslow, UK) diluted in radioreceptor assay buffer (RAB; 83 mM NaCl, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.6). The final concentration of radiolabeled progesterone was approximately 5 nM. Tubes were preincubated on wet ice for 15 min before addition of 150 µl freshly thawed OPM, diluted 1:1 in RAB, and then incubated for a further 90 min at 4°C. Bound/free separation was achieved by vacuum filtration of 250 µl of the incubate from each tube through a glass fiber filter (GF/B; Whatman International, Maidstone, UK), which had been presoaked in ice-cold RAB for 2 hr. Each filter was washed through with 10 ml of ice-cold RAB, and bound [^{3}H]progesterone was measured on a Hewlett-Packard Tricarb liquid scintillation analyzer. Nonspecific binding was estimated by incubating tracer and competitor vehicle with RAB alone; this value was subtracted from all other values.

Statistics. Where indicated, the percentage GVBD for different treatments was analyzed by one-way analysis of variance (ANOVA) using the SPSS statistical software package (SPSS Inc., Chicago IL). Differences between treatments were assessed using Bonferroni's test and were defined as significant when $p<0.05$.

Results

Dose response of progesterone-induced GVBD. Increasing doses of progesterone stimulated an increasing proportion of *Xenopus* oocytes to undergo maturation, as determined by GVBD. The sensitivity of *Xenopus* oocytes to GVBD varies considerably between frogs: in our assay the median effective concentration (EC_{50}) for GVBD ranged from 2.5 to 400 nM over 20 experiments, with a mean of 72.6 nM. This variability may result from differences in endogenous gonadotropin levels that sensitize preovulatory oocytes to progesterone induction of GVBD (*37*). Consequently, in each experiment, oocytes from a single frog

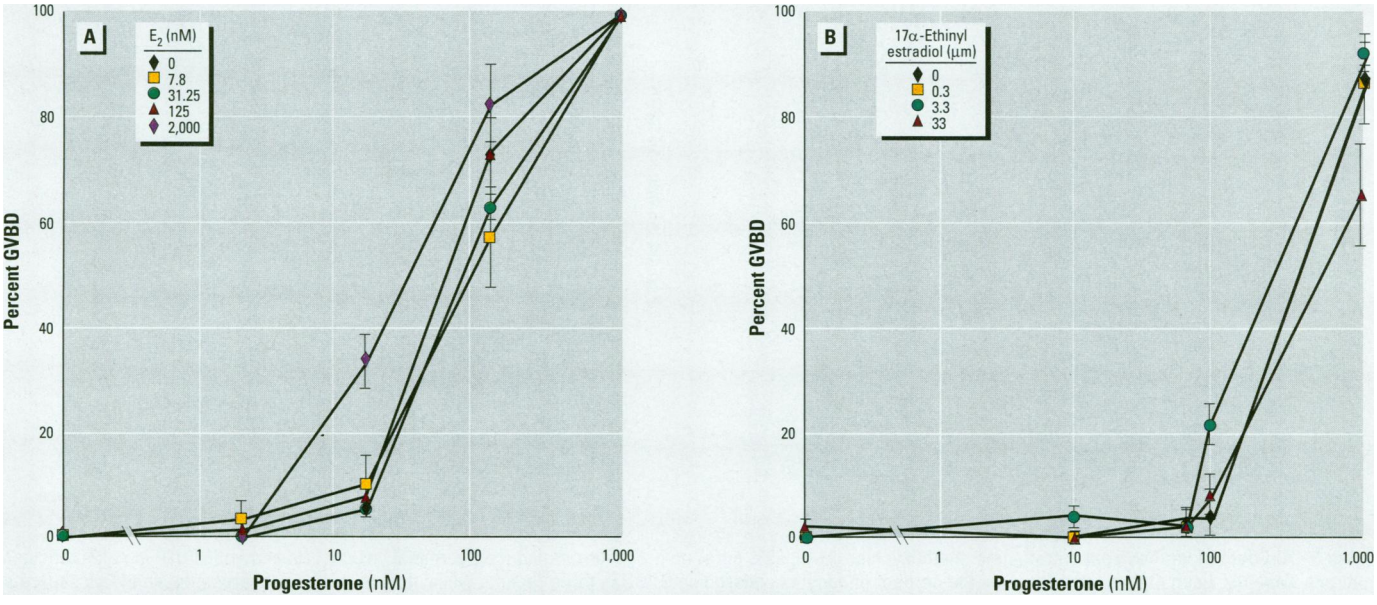

Figure 2. Effect of various concentrations of (A) 17β-estradiol (E_2), with 8-hr pretreatment and (B) 17α-ethinyl estradiol with 16-hr pretreatment on progesterone-induced germinal vesicle breakdown (GVBD) in naked *Xenopus* oocytes. Data points represent the mean percent of GVBD in three replicate wells, 20 oocytes/well; error bars represent 1 standard error.

were used, and all experiments included positive and negative controls.

Effects of estrogens. The natural and synthetic estrogens E_2 and 17α-ethinyl estradiol had no observable effect on GVBD in the dose range 5 nM–5 µM, when added to culture media at the same time as progesterone (data not shown). Because these findings contrast with earlier reports of antagonistic effects of estrogens on amphibian oocyte maturation (*28,30*), we performed pretreatment experiments based on these reports. Pretreatment with 2 µM E_2 for 8 hr appeared to weakly agonize GVBD (Fig. 2A), whereas with 16-hr pretreatment 17α-ethinyl estradiol exhibited weak agonistic (at 3 µM) and antagonistic (at 33 µM) effects on progesterone-induced GVBD (Fig. 2B).

Table 1. Effects of endocrine-disrupting contaminants (EDCs) on frequency of progesterone-induced germinal vesicle breakdown (GVBD)

	Concentration (nM)		
Chemical	62.5	250	4,000
Bisphenol A	93.14	93.38	105.32
Octylphenol	95.60	97.80	100.00
Di-*n*-butyl phthalate	101.48	97.34	106.26
o,p'-DDT	101.40	101.51	101.83
Methoxychlor	94.8	76.35	32.93

Values shown are mean percentage GVBD induced by progesterone (125 nM) in the presence of various EDCs at three concentrations. Values are normalized as percentage of GVBD observed in control (progesterone 125 nM and vehicle only), and represent triplicate determinations with oocytes from one frog for each compound.

Effects of endocrine-disrupting contaminants. The estrogenic EDCs *o,p'*-DDT, octylphenol, di-*n*-butyl phthalate, and bisphenol A had no observable effect on GVBD stimulated by progesterone (1.95–1,000 nM) in the dose range 62.5–4,000 nM, when added to media at the same time as progesterone (Table 1). Pretreatment experiments were not performed with these compounds.

In contrast, methoxychlor (95%), a proestrogenic organochlorine pesticide (*38*), caused a highly significant, dose-dependent inhibition of GVBD (Table 1, Fig. 3A). Increasing concentrations of methoxychlor caused a rightward shift of the GVBD dose response to progesterone (1.95–1,000 nM). The inhibitory effect of methoxychlor was overcome at a high dose of progesterone (1 µM), indicating that methoxychlor was not blocking GVBD through general toxicity to the oocyte (Fig. 3A). To determine the potency of this inhibition, we estimated the mean methoxychlor concentration required to cause 50% inhibition of progesterone-induced GVBD (IC_{50}) in five replicate experiments using oocytes from five different frogs. We corrected for the variable sensitivity of oocytes to progesterone among frogs by estimating the percent of inhibition at increasing concentrations of methoxychlor, at the progesterone EC_{50} for each experiment. This yielded an IC_{50} value for methoxychlor of 72 nM at an EC_{50} for progesterone that averaged 7.5 nM in these experiments (Fig. 3B).

Mechanism of methoxychlor inhibition of GVBD. GVBD was also inhibited by highly purified methoxychlor (99.25% pure), to an extent similar to that achieved with the 95% preparation, whereas methoxychlor's metabolite HPTE did not inhibit oocyte maturation (Fig. 4). These results indicate that the inhibitory activity of methoxychlor is not due to contaminants or conversion by the oocyte to HPTE, to which its estrogenic activity is attributed (*39*). These findings, combined with the lack of significant inhibition of GVBD by the potent estrogens E_2 and 17α-ethinyl estradiol, suggest that the activity of methoxychlor in this assay is nonestrogenic in nature.

To test this hypothesis, we assessed the ability of methoxychlor (95%) to inhibit GVBD induced by a single nonmaximal dose of progesterone (31.25 nM) in the presence of the potent estrogen receptor antagonist ICI 182,780 (*40*). Dose-dependent inhibition of GVBD by methoxychlor was unaffected by ICI 182,780 at concentrations up to 1 µM (Fig. 5), indicating that methoxychlor inhibition of GVBD is not mediated by the estrogen receptor. The ICI compound exhibited no innate activity in inducing oocyte maturation in the absence of progesterone (data not shown).

Because methoxychlor antagonized the maturation-inducing effect of progesterone in this assay, we compared its activity to that of the synthetic antiprogestins RU 486 and

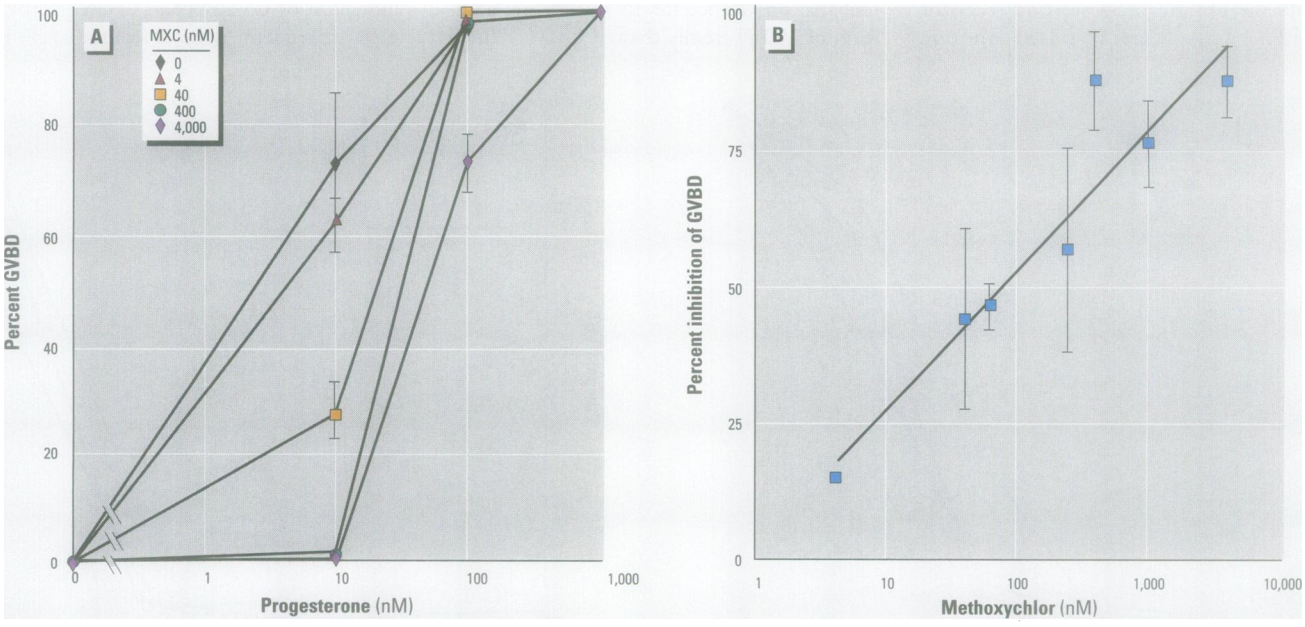

Figure 3. (A) Effect of various concentrations of methoxychlor (MXC; 95% pure) on progesterone-induced germinal vesicle breakdown (GVBD) in naked oocytes from one *Xenopus*. Each data point represents the percent of oocytes undergoing GVBD in three replicte wells; these results are representative of five separate similar experiments using oocytes from five different frogs. Error bars represent 1 standard error (SE). (B) Mean percent inhibition of GVBD stimulated by progesterone at EC_{50} (dose that stimulates 50% GVBD; mean 7.5 nM) at various concentrations of methoxychlor (95%). Each data point represents the mean of five separate experiments using naked oocytes from five different frogs; error bars represent 1 SE. Mean IC_{50} (dose that inhibits 50%) for methoxychlor in these experiments is 72 nM.

ZK 98.299 (*41,42*). These did not exhibit any inhibitory activity in the dose range 0.1–25 µM (Fig. 6). Neither compound was capable of inducing GVBD in the absence of progesterone, although ZK 98.299 appeared to be agonistic in a dose-dependent manner in the presence of progesterone (Fig. 6B).

We also performed experiments to try to determine when methoxychlor exerts its action. GVBD induced by 100 nM progesterone (EC_{50}) is significantly inhibited in oocytes co-exposed to methoxychlor (4 µM). However, this effect becomes nonsignificant when methoxychlor exposure is delayed 2 hr after addition of progesterone (Fig. 7). For comparison, GVBD was stimulated with a 2-hr exposure to progesterone, followed by washout. The percent of GVBD stimulated by this was not different from that observed in the delayed-methoxychlor treatment group. This suggests that methoxychlor targets events occurring in the first 2 hr after exposure to progesterone: no maturational signaling initiated in the first 2 hr of incubation was blocked by subsequent exposure to methoxychlor. Washout of methoxychlor was ineffective because maturation was inhibited to the same extent in the oocytes exposed to methoxychlor for 2 hr followed by washout as in oocytes pretreated with methoxychlor for 2 hr before progesterone, but with no washout (Fig. 8).

Radioreceptor assay. We examined the ability of methoxychlor and its estrogenic metabolite HPTE to interact with the omPR through competitive displacement of radiolabeled progesterone. Neither compound exhibited any competitive binding affinity for the omPR in this assay, relative to progesterone. Binding of radioactive progesterone was not displaced by methoxychlor or HPTE at a concentration of 10 µM, rather both compounds increased binding to around 120% of the control value (Fig. 9). Mean displaceable binding of progesterone in this assay, i.e., binding of radiolabeled progesterone, which could be displaced by the presence of an excess of cold progesterone (10 µM), was 62% of total binding (Fig. 8).

Discussion

Using an *in vitro* GVBD assay, we have shown that progesterone-induced maturation of *Xenopus laevis* oocytes is not sensitive to estrogens, but is potently inhibited by the pesticide methoxychlor. This activity appears to be essentially nonestrogenic for the following reasons: natural and synthetic estrogens had no significant antagonistic effect on GVBD; structurally related xenobiotics with innate estrogenic activity (e.g., *o,p′*-DDT) were inactive; the methoxychlor metabolite HPTE, to which the *in vivo* estrogenicity of methoxychlor is attributed (*38*), was also inactive; and methoxychlor inhibition of GVBD was not antagonized by the estrogen receptor antagonist ICI 182,780.

Earlier observations on the effects of estrogens on amphibian oocyte maturation are equivocal: pretreatment with estrogens has been shown to both agonize and antagonize progesterone-induced maturation of *Xenopus* oocytes, and estrogens may be considered weak agonists/antagonists in this system (*28,43*). Our findings are consistent with this, as E_2 and 17α-ethinyl estradiol exhibited weak agonist activity at low micromolar concentrations, and at higher doses (33 µM), 17α-ethinyl estradiol was slightly antagonistic. Consequently, antagonism of progesterone-induced GVBD using naked *Xenopus* oocytes offers little potential as an assay for functional estrogenicity of xenobiotics in amphibia, as has been suggested (*44*). However, this does not imply that other types of GVBD assays may not be useful in this respect. For example, gonadotropin-induced GVBD in follicle cell-enclosed oocytes from *Rana pipiens* is strongly antagonized by estrogen without pretreatment (*30,45*). The mechanism of this antagonism appears to be feedback inhibition of follicle cell 3β-hydroxysteroid dehydrogenase activity (*46*), which is involved in generating the paracrine progesterone signal that stimulates GVBD in response to gonadotropin (*47*). A two-cell model of gonadotropin-induced GVBD of follicle cell-enclosed oocytes, by virtue of incorporating steroidogenic machinery, might

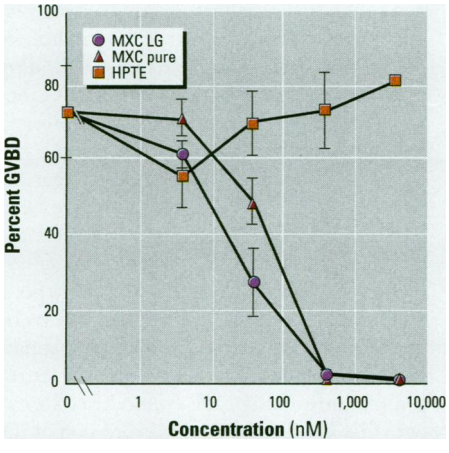

Figure 4. Effect of laboratory grade methoxychlor (95%; MXC LG), pure methoxychlor (99.25%; MXC pure), and the methoxychlor metabolite 2,2-bis(*p*-hydroxyphenyl)-1,1-trichloroethane (99.21%; HPTE) on germinal vesicle breakdown (GVBD) induced by 10 nM progesterone in naked *Xenopus* oocytes. Data points represent the mean percent of oocytes undergoing GVBD in three replicate wells, with 20 oocytes/well; error bars represent 1 standard error.

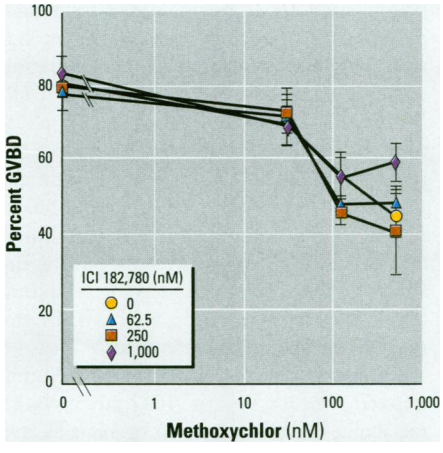

Figure 5. Effect of various concentrations of the estrogen receptor antagonist ICI 182,780 on methoxychlor inhibition of germinal vesicle breakdown (GVBD) induced by 30 nM progesterone in naked *Xenopus* oocytes. Each data point represents the mean percent of oocytes undergoing GVBD in four replicate wells, with 20 oocytes/well; error bars represent 1 standard error.

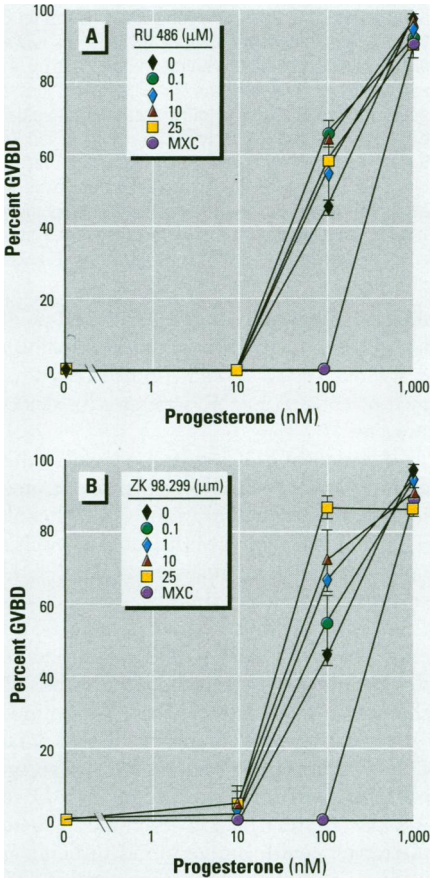

Figure 6. Effect of various concentrations of the antiprogestins RU 486 (A) and ZK 98.299 (B) on progesterone-induced germinal vesicle breakdown (GVBD) in naked *Xenopus* oocytes. These results are representative of two similar experiments. MXC, methoxychlor. Data points represent the mean percent of oocytes undergoing GVBD in three replicate wells, with 20 oocytes/well; error bars represent 1 standard error.

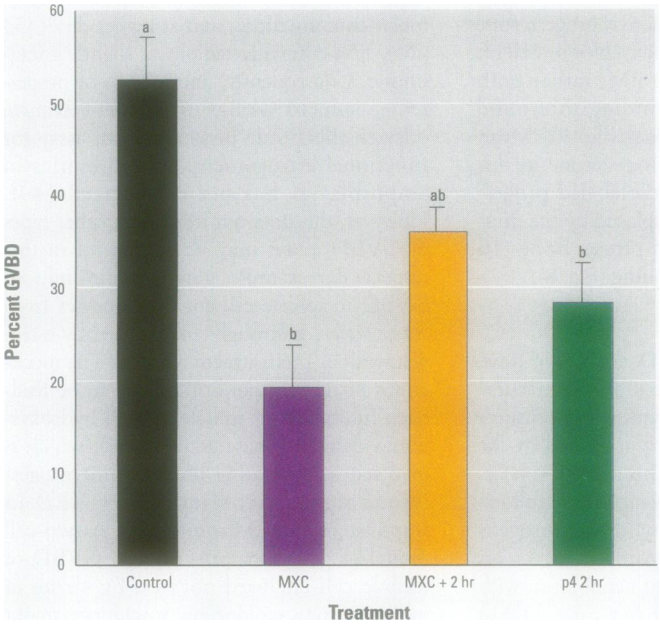

Figure 7. Effect of methoxychlor on germinal vesicle breakdown (GVBD) induced by progesterone at a concentration close to its EC_{50} (median effective concentration) in naked *Xenopus* oocytes. Oocytes were exposed to progesterone (100 nM) for 24 hr and either vehicle alone (Control), 4 µM methoxychlor added at the same time as progesterone (MXC), or 4 µM methoxychlor added 2 hr after progesterone (MXC + 2hr). A fourth treatment involved exposure of oocytes to progesterone (100 nM) for only the first 2 hr of the 24-hr incubation period; progesterone was then washed out and oocytes were incubated in medium and vehicle (EtOH) alone for the remainder of the experiment (p4 2 hr). Each bar represents the mean percent of oocytes undergoing GVBD in three replicate wells, with 20 oocytes/well; error bars represent 1 standard error. Bars marked with different letters are significantly different at $p<0.05$.

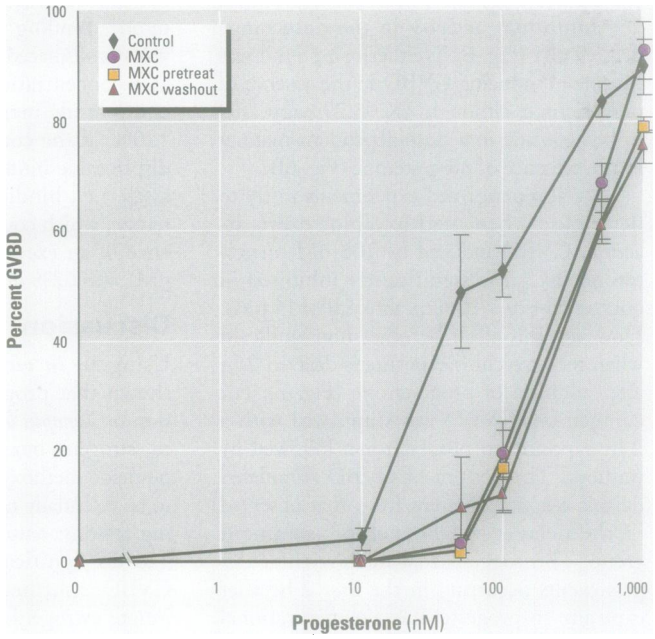

Figure 8. Effect of pretreatment or washout on methoxychlor inhibition of progesterone-induced germinal vesicle breakdown (GVBD). Naked *Xenopus* oocytes were exposed for 24 hr to either progesterone (various doses to give dose–response curve) and vehicle (Control), or progesterone and 4 µM methoxychlor (MXC). Some oocytes were exposed to 4 µM methoxychlor 2 hr before addition of progesterone without washout (MXC pretreat), or alternatively, were exposed to 4 µM methoxychlor for 2 hr, which was then washed out prior to addition to progesterone (MXC washout). Each data point represents the mean percent of oocytes undergoing GVBD in three replicate wells, with 20 oocytes/well; error bars represent 1 standard error.

therefore represent a more sensitive end point for estrogenic activity in the adult amphibian ovary. Such assays should also be assessed for their potential as amphibian screens for xenoestrogens.

A variety of EDCs were also tested in this assay to assess their effect on progesterone-induced oocyte maturation. Octylphenol, *o,p'*-DDT, di-*n*-butyl phthalate, and bisphenol A are environmentally persistent chemicals that exhibit innate estrogenic activity (*7,48–50*). Given the lack of significant effects of natural and synthetic estrogens in this GVBD assay, it is perhaps not surprising that these weakly estrogenic compounds were inactive. It is interesting to note that DDT has previously been reported to antagonize progesterone-induced GVBD in *Xenopus* oocytes (*35*). However, the apparent discrepancy with our results is difficult to interpret because it is not clear which DDT isomer was used or at what concentration.

In contrast, methoxychlor antagonized progesterone quite potently, inhibiting GVBD with an IC_{50} of approximately 72 nM, at the EC_{50} dose of progesterone. The organochlorine pesticide methoxychlor is a *p,p'*-methoxy derivative of DDT, which has low toxicity to mammals and low persistence and bioaccumulation in the environment (*51,52*). Consequently, it has been used extensively in place of banned pesticides such as DDT and chlordecone (kepone) to the present day (*21*). In use to control black flies, methoxychlor has been applied to river systems or canals, generally at a concentration of 0.3 mg/l in 7.5- or 15- min pulses (*53*). Measurements downstream of such applications (120 km) have detected peak concentrations of 1.4 ppb (*54*). These environmental levels of methoxychlor equate approximately to concentrations of 870 nM (application) and 4 nM (downstream), putting the IC_{50} of 75 nM for methoxychlor inhibition of GVBD reported here well within the range of environmental relevance.

Methoxychlor is a proestrogen, requiring hepatic conversion to the hydroxylated metabolite HPTE to exert estrogenic effects *in vivo* (*38*). While it is unlikely that oocytes in meiotic arrest are able to metabolize methoxychlor, we tested HPTE in the GVBD assay and found it to be inactive. Moreover, because some of the estrogenic activity of technical and laboratory grade methoxychlor has been attributed to base-soluble contaminants (*39,55*), we also tested a highly purified sample (*56*). Pure methoxychlor also inhibited GVBD, to an extent similar to 95% methoxychlor, indicating that methoxychlor, rather than contaminants or metabolites, is inhibiting GVBD in our assay. These findings are consistent with our contention that methoxychlor activity in this system is not correlated with estrogenicity per se, and this is supported by our finding that methoxychlor inhibition of GVBD is not antagonized by the presence of the estrogen receptor antagonist ICI 182,780.

Estrogenic effects of xenobiotics that are not antagonized by ICI 182,780 have been reported in other systems. For example, the estrogenic activities of a catechol estrogen (4-hydroxyestradiol-17β; 4-OH-E_2) and kepone in the mouse uterus have been shown to be mediated by an alternative pathway, apparently independent of classical nuclear estrogen receptors (ERs) (*57*). In the ERα knockout (ERKO) mouse, uterine expression of the estrogen responsive lactoferrin gene is upregulated by 4-OH-E_2 and kepone (but not E_2). This ERα-independent response to these compounds is not inhibited by ICI 182,780, indicating that these effects are also independent of ERβ and may be mediated by a distinct and novel estrogen-signaling pathway (*58*). Similar effects have also been observed in ERKO mice with methoxychlor (*59*).

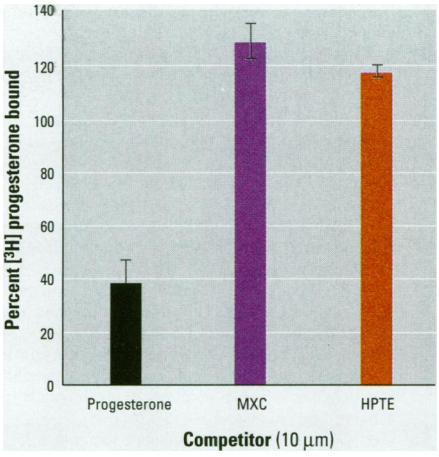

Figure 9. Relative binding affinity (RBA) of progesterone, pure methoxychlor (MXC), and pure 2,2-bis(*p*-hydroxyphenyl)-1,1-trichloroethane (HPTE) to *Xenopus* oocyte plasma membrane (OPM). Percent of binding of radiolabeled progesterone was determined in the presence of 10 μM of each compound, relative to radiolabeled progesterone and DMSO vehicle only. Each bar represents the mean RBA of each compound from quadruplicate determinations in three separate experiments using OPM prepared from three different frogs; error bars represent 1 standard error.

Given that none of the existing reports of estrogenic inhibition of GVBD define a mechanism for such effects (*28,30*) and the mechanism of methoxychlor inhibition of GVBD is still unclear, it remains possible that methoxychlor is exhibiting similar novel estrogenic activity in this system. However, it should be noted that the ERα/ERβ-independent activity of methoxychlor in the ERKO mouse is expressed *in vivo*, where it is, presumably, converted to active estrogenic metabolites. In contrast, the apparently nonestrogenic activity of methoxychlor in inhibiting GVBD that we describe is expressed *in vitro*. The failure of HPTE to similarly inhibit GVBD indicates that methoxychlor is not metabolized to HPTE by the oocyte, and is expressing an innate activity. Furthermore, while evidence exists that methoxychlor can interact with both ERα and ERβ (*60*), the very poor affinity of this interaction is not consistent with the potency of methoxychlor in inhibiting GVBD.

The diversity of steroids active in inducing oocyte maturation and the transcriptional independence of the process are not consistent with a genomic mechanism involving a nuclear hormone receptor (*28*). The mechanism of progesterone action is essentially cell surface mediated, as early events in maturation involve changes in cAMP (adenosine 3′,5′-cyclic monophosphate) machinery and Ca^{2+} fluxes (*35*), and a progesterone receptor in the oocyte plasma membrane has been identified and characterized by several groups (*36,61,62*). Methoxychlor apparently interferes with early events in progesterone-induced maturation, as delay of methoxychlor treatment 2 hr after progesterone exposure resulted in a reduced inhibitory effect on GVBD. This suggests that methoxychlor is not blocking downstream events, such as formation of maturation promoting factor, which occurs around 4–6 hr after progesterone initiation (*35*).

A number of xenobiotics have been shown to interact with progesterone receptors (*63*); HPTE has in fact been shown to interact with estrogen, androgen, and progesterone receptors (*64*). We hypothesized, therefore, that methoxychlor may inhibit GVBD by interfering with the initial interaction of progesterone with its cell surface receptor. However, 2 hr exposure to methoxychlor could not be washed out: GVBD induced by subsequent exposure to progesterone was inhibited to the same degree as achieved with methoxychlor pretreatment. While this could mean that methoxychlor has an irreversible effect on the oocyte, this is not consistent with the ability of high (1 μM) concentrations of progesterone to overcome its effect. The washout may have been insufficient to remove methoxychlor, which might accumulate in the oocyte due to its lipophilic nature. Indeed, attempts at competitive binding experiments with progesterone and antagonists with intact oocytes have generally been unsuccessful, owing to the large uptake and nonspecific retention of steroids by the yolk (*28*).

The activity of methoxychlor in this assay was not comparable to that of synthetic antiprogestins, as RU 486 and ZK 98.299 did not antagonize progesterone-induced GVBD. This may reflect the fact that these compounds were developed as antagonists of the mammalian nuclear progesterone receptor, rather than an amphibian omPR. The functional steroid specificity of oocyte maturation is considerably wider than those of classical nuclear hormone receptors. Moreover, the synthetic progestin analog R5020, which also acts as an agonist in GVBD (*28*), has a low affinity for the omPR as determined in a competitive binding assay using oocyte plasma membranes (*36*). Using the same competitive binding assay, we found that neither methoxychlor nor its metabolite HPTE were able to displace specific binding of radiolabeled progesterone to its receptor in the plasma membrane. In summary, it seems unlikely that methoxychlor is exerting its inhibitory activity in GVBD through direct competition with progesterone for the omPR. Other potential mechanisms for methoxychlor action include noncompetitive binding to the omPR, altering omPR–adenylate cyclase interaction; interference with progesterone- induced suppression of adenylate cyclase activity; changes in cAMP levels through modulation of phosphodiesterase activity; and membrane disruption resulting in altered Ca^{2+} fluxes.

The action of progesterone in stimulating maturation in amphibian oocytes is one of many examples of nongenomic effects of progesterone and other sex steroids on target cells (*65,66*). Although a number of such effects appear to be mediated through heterologous hormone receptors (*67*) or novel membrane receptors (*68–70*), little attention has been paid to the potential for endocrine-disrupting effects of xenobiotics through these alternative mechanisms (*70*). Specific oocyte membrane receptors for maturation-inducing progestins have been identified and characterized in other fish species (*71,72*) and may be similar and related to the *Xenopus* omPR. Xenobiotic inhibition of progestin-induced GVBD has already been demonstrated in Atlantic croaker oocytes (*27*). We have now shown that the proestrogenic pesticide methoxychlor is also capable of potently inhibiting the nongenomic effects of progesterone on *Xenopus* oocytes at concentrations that are of environmental relevance. These findings add to evidence that any steroid hormone-regulated process, including those operating through nongenomic mechanisms, is a potential target of endocrine-disrupting contaminants. This highlights the need for a broad-based and flexible approach to screening of environmental contaminants for endocrine-disrupting activity.

We are currently investigating *in vivo* effects of methoxychlor on oogenesis in *Xenopus laevis* to determine whether the potent *in vitro* effect on oocyte maturation described here translates to reproductive dysfunction at the level of the whole organism. Such an effect would have profound implications for the impact of methoxychlor, or compounds with similar activity, on amphibian reproductive physiology.

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